The Attenuated Total Reflectance Infrared Microspectroscopy

of Bacterial Colonies on Agar Plates

An Honors Thesis (HONRS 499)

By

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May 1999

Graduate May 1999

Explanation of Paper—Purpose

This paper is an article that will be submitted for publishing to a science journal sometime in the future. The article is a summary and the culmination of a year of research in the department of chemistry under Dr. Patricia Lang. My research was made possible through the Undergraduate Fellows Program and the Honors College. The bulk of the project was done during the 1997-1998 school year and completed in the summer of 1998.

The topic of my study was the prediction of the Gram stain property of bacteria using the methods of infrared (IR) spectroscopy and a statistical analysis called Principal Components Regression. Bacteria cultures were streaked onto agar plates and then incubated for 24 hours for optimum growth. Spectra were then obtained from the bacteria while still on the agar using an infrared microscope fitted with a special objective, the attenuated total reflectance objective (ATR). This objective has a crystal in it that acts as a prism. Light enters through the crystal, bounces off the colony, and is reflected back through the crystal into the microscope. Bacteria absorb light from the infrared region of the electromagnetic spectrum. If IR beams are shined on a colony through a microscope fitted with an ATR objective, a machine reads the frequencies of the different absorptions of the reflected light and can produce a picture, or spectrum, of these absorptions. This spectrum is, in essence, a fingerprint of the bacteria colony. Statistical analyses can

then be used to predict a certain property of the bacteria species through establishing correlations between spectra and a known calibration set of factors, or principal components, which can be combined to recreate the original spectra. These factors are similar to spectra themselves, and the number of factors is equal to the number of bacteria samples in the set, but some are not significant and are discarded. The significant factors are the principal components. Each original spectrum can be represented as a set of coefficients, or components, which correspond to the contribution of each factor to that spectrum. Correlations are established between the principal components and the spectra, and a statistical model is built. Using this model, one can predict a certain property from the spectrum, and it can then be used to analyze unknown samples of bacteria. The property we examined is Gram stain of bacteria. The idea is that one can predict the Gram stain property of an unknown bacteria using the techniques of ATR spectroscopy and Principal Components Regression analysis.

Abstract

The attenuated total reflectance spectra of 44 species of bacteria were obtained while still on the agar using the infrared microscope. Compared to the specular reflectance spectra of the same species, the $\nu(OH)$ and $\delta(OH)$ absorptions are not broad and totally absorbing due to the reduced pathlength resulting from using the ATR objective. The ATR spectra show slight frequency shifts in bands from the specular reflectance spectra. These shifts are consistently higher in the ATR spectra, except for the $\nu(PO_2^-)$ band and the C-O-C and C-O-P stretching bands which are lower. Principal components regression analysis was used to obtain a measure of differentiating potential of our ATR spectra. A positive one, +1, was assigned to Gram positive species and a negative one, -1, was assigned to Gram negative species. The prediction results after performing a cross validation show that Gram positive bacteria have positive predicted values for Gram stain while Gram negative bacteria have negative values. Also, it was found that ATR spectra can be obtained from bacteria grown on more than 1 type of agar and still have good differentiating capabilities for Gram stain using PCR analysis. Overall, using ATR spectra from colonies directly on the agar provides significantly better differentiating spectral information and predicting capabilities than specular reflectance spectra.

Introduction

The differentiation and identification of bacteria using infrared spectroscopy was first reported in the 1950's and 1960's (1,2). It was shown that bacteria exhibit infrared spectra unique to individual strains, but the differences are subtle. Infrared spectroscopy was seen as possible but not practical due to the rigid control needed in growing and sampling conditions.

In 1988, Naumann once again looked at the characterization of bacteria by IR spectroscopy, but by using Fourier Transform IR spectroscopy and statistical methods (3). This factor analysis clustered the spectra in 2 different groups corresponding to Gram-negative and Gram-positive species. In 1992, he added the use of the infrared microscope to his study (4). In all of these past studies, transmittance spectra on dehydrated bacterial films were used in the data set.

By collecting reflectance spectra from colonies still on the agar, however, sample preparation would be eliminated and more selective spectral information might be obtained than when using transmittance spectra of dehydrated films. This is due to the make-up of the bacterial cell wall. In Gram-positive bacteria, the cell wall is composed of several layers of peptidoglycan, a mixture of polysaccharides and polypeptides, and in Gram-negative bacteria, the cell wall is made up of a thin layer of peptidoglycan covered with a lipid-rich outer coat containing hydrophobic proteins (5). If the bacteria cultures are dehydrated, the structure may change, thereby removing some differentiating features.

Dr. Lang and Susan Sang investigated this possibility of specular reflectance of intact bacteria (6). They used the IR microscope fitted with a Cassagrain lens to obtain spectra. Light is directed through the lens to the colony where some is reflected. Light then returns through the lens and is detected by an IR detector. Principal Components Regression Analysis was then used

to analyze the Gram stain property of the bacteria.

Lang and Sang's results on specular reflectance microspectroscopy of bacteria shows that meaningful spectra can be obtained *in situ* and without sample preparation. It can also be concluded that factor analysis using specular reflectance spectra has the same or slightly better prediction capabilities as the method using transmittance spectra. One problem with specular reflectance is the fact that pathlength cannot be controlled and that totally absorbing stretching and bending OH bands are obtained.

The solution to the problem of pathlength and the foundation of our work is to use an Attenuated Total Reflectance objective on the microscope to obtain spectra of intact bacterial colonies. The ATR objective reduces the pathlength and allows one to two microns on the surface of the colony to be probed. The ATR method brings the sample into contact with an internal reflection element with a higher refractive index than the sample. If the incident IR radiation sent into the internal reflection element is greater than or equal to the critical angle, the radiation will be totally internally reflected. When using the ATR crystal in spectroscopy, the internal reflectance occurs at contact, but a small amount of radiation does penetrate a short distance into the sample. The wavelengths of light absorbed by the sample are absent in the reflective beams, which are measured and converted to ATR spectra.

The ATR spectra are the analyzed using a factor analysis called Principal Components Regression analysis. PCR uses a statistical approach to establish correlations between spectra and known properties of a calibration set. It generates a series of factors, or principal components, which can be combined linearly to recreate the original spectra. The factors are much like spectra themselves, except they can be positive or negative. The number of factors is equal to the number of samples in the set, but many account for noise and are discarded. The

significant factors are called principal components. Multiple Linear Regression is used to establish correlations between the principal components and the spectra. Once the model is built, one can predict the property from the spectrum using the regression equation. The model can then be used to analyze unknown samples. We analyzed Gram stain property in our approach.

Experimental

All specular reflectance infrared spectra were obtained using a Perkin-Elmer 1760-X Fourier Transform infrared (FT-IR) spectrometer fitted with a Spectra-Tech IR Plan I. A mercury cadmium telluride detector with a detector area of .25 mm by .25 mm was used. Infrared reflectance measurements of the bacterial colony samples were acquired for each spectrum at 16 cm^{-1} resolution, a gain of 8, and 100 signal averaged scans.

An Attenuated Total Reflectance (ATR) objective was used with the FT-IR microscope to obtain specular reflectance spectra of the bacterial colonies while still on the agar *(in situ)*. The objective is a tri-mode objective with a dual magnification of 15X/25X (Spectra-Tech ATR Objective Users Manual Version 2.4), as Figure 1 shows. The crystal in the objective through which light passes is made of Zinc Selenide (ZnSe) and acts as a prism to reflect the light off a 1-2 micron area on the surface of the colony. The three modes of the objective are the Survey Mode (Figure 1A), the Contact mode (1B), and the ATR mode (1C). The Survey mode allows viewing of the sample from a distance of 3 mm in order to center the sample in the field of view. Visible light is sent from the microscope through an internal aperture in the objective in a single cone of light, so that the sample may be viewed directly. The Contact mode is used to bring the sample into contact with the crystal, so that light may be reflected off the surface of the sample back through the microscope and into the FT-IR spectrometer. Visible light is separated into two beams by the internal aperture of the Contact mode and converges in the objective at an almost 90° angle. When viewed on a video screen, this optical path makes it possible to see contact occurring between the objective and the sample. The ATR mode is used when performing an experiment. Infrared light is sent through the internal aperture of the ATR mode in two cones

and is reflected within the objective, so that the infrared light beam is at a 45° (+/- 5°) angle to the sample. This allows for optimum reflectance off the surface of the sample. See Figure 1 for diagrams of the light paths through the objective. An upper 2.5-mm aperture was used, so that an ideal viewing window was attained and maximum energy collected for the IR spectrum of the sample.

The preparation of bacteria cultures was according to standard methods (7). Bacteria standard samples were provided by the Ball State Center for Medical Education and stored in liquid broth at -78°C. The bacteria were transferred from the thawed broth, streaked onto tryptic soy agar plates, and then incubated at 37°C overnight. In another experiment, bacteria were streaked onto blood agar plates, then incubated at 37° overnight, the same as the T-soy agar plates. Blood agar was made according to standard methods. The plates were removed after 24 +/- 2 hours in the incubator, and the specular reflectance infrared spectra were obtained as described below. The plates were allowed to sit open for 2 to 3 minutes before obtaining spectra. This prevents fogging of the crystal, which makes it difficult to determine contact with the bacterial colony in Contact Mode.

The ATR spectra of the bacterial colonies were obtained *in situ*, with the bacteria still on the agar and the agar still in the plate. The plate was placed on the FT-IR microscope stage and the objective placed in Survey mode. The stage was raised until the light through the objective became the size of a pinpoint, then the light was centered on a bacterial colony. The stage was brought up until 3 mm (+/- 1 mm) separated the crystal and the colony. The bacteria colony was observed to come into view on a video screen, evident as a change in brightness in the field of view. The slide selector was changed to Contact mode. Using the coarse focus knob and

viewing from the side, the stage was raised until the sample was almost touching the crystal. While watching the video screen, the fine focus knob was used to bring the sample into contact with the crystal, which was evident as a change in the field of view from brightness to black. The slide selector was then switched to ATR mode and the light in the microscope from View to IR Reflectance mode. A single-beam spectrum of the colony was collected and was ratioed against a single-beam background spectrum. The single-beam spectrum was obtained through the ATR element when it was not in contact with the sample.

Once a spectrum was obtained, the stage was lowered, the nosepiece rotated so the ATR objective was facing outward, and the crystal was cleaned. The optimum cleaning solutions are a 10% bleach solution, distilled water, and acetone. The crystal is wiped clean with a cotton swab soaked with the bleach solution to kill the bacteria. A piece of lens paper moistened with distilled water is used to remove any excess bacterial film, and then acetone is applied to the crystal with lens paper to facilitate dryness.

The principal components regression analysis was performed using the Perkin-Elmer software, Quant +, on the ATR reflectance spectra obtained as described by the above procedure. The average of three spectra obtained from separate colonies on three different days comprised each standard. In order to obtain some measure of the differentiating potential of the spectra, we assigned a +1 for a Gram positive property and a -1 to a Gram negative property to each averaged ATR spectrum. The data analysis was run using a spectral range of 4000 to 700 cm^{-1}, with a blank region from 2500 to 2200 cm^{-1} to eliminate carbon dioxide absorptions. The scaling, normalization, smoothing, and baseline were set at zero. The minimum number of factors was set at two and the maximum at 15. The expert assist option was used with the following predefined set of threshold values: F-test was set at 10%; backward search at 25%;

outliers, 10%; influence ratio, 10%; validation leverage ratio at 1.0; and the residual ratio at 3.0.

A cross validation was performed to see how closely the Gram stain property could be predicted for each standard after its spectrum was removed from the calibration set. Nine factors, or principal components, were extracted for the T-soy models in the analysis and ten factors were extracted for both blood models. Only 7 of these factors correlated to Gram stain property in all of the models.

Results and Discussion

The main question examined and the purpose for our study was whether the use of the ATR microscope objective would produce the same or better prediction capabilities for Gram stain property than the methods using specular reflectance. Figure 2 (top) shows the ATR spectrum of *Enterococcus faecalis*, a Gram positive species of bacteria, and the ATR spectrum of *Shigella flexneri* (bottom), a Gram negative species. As seen in the figure, the protein components of the bacteria dominate the spectrum between 2000 and 700 cm^{-1}, indicated by the intense $v(C=O)$, amide I band, and the $\delta(C-N-H)$, amide II band (labeled A and B, respectively). This is consistent with the specular reflectance spectra of the same two species. Also observed in the spectra are the methylene/methyl bending (band C); methyl bending and/or symmetric COO$^-$ stretching of carboxylates (band D); PO$_2^-$ asymmetric stretching of phosphodiesters (band E); and C-O-C and C-O-P stretching of polysaccharides (band F) (8).

If these spectra are compared to the previously published specular reflectance spectra (6), we see the same number and types of bands, but a comparison of frequencies show slight shifts, as seen in Table 1. The ATR shifts are consistent between species for all bands except the amide I, which is superimposed by $\delta(OH)$. The amide II (band B) is always higher in the ATR spectrum for both species than in the specular reflectance spectra; the methylene/methyl (band C) bands are higher in the ATR spectrum, as well as the COO$^-$ stretching bands (band D). The PO$_2^-$ stretching (band E) is higher in frequency in the specular reflectance spectrum for both species, as is the C-O-C and C-O-P stretching (band F) of polysaccharides. One can also note that although the $v(OH)$ and

δ(OH) absorptions are intense in the ATR spectra, they are not broad and totally absorbing as in the previously published specular reflectance spectra.

Principal Components Regression (PCR) was used to analyze the averaged ATR spectra of the original 31 bacteria species used in the specular reflectance study and predict the correlation of Gram stain property. The results of our experiments were promising. All of the Gram positive species of our original set had positive predicted values for Gram stain, ranging from .21 to 1.30. The Gram negative species had negative predicted values for Gram stain, ranging from -.57 to –1.30, as shown in Table 2. This shows an obvious way for differentiating between Gram negative and Gram positive species, specifically that positive values indicate positive species, while negative values indicate negative species. The use of PCR in our experiment shows potential in its simplicity, as it can replace a two-dimensional cluster plot with a one-dimensional grouping. The simplified output provides an easy comparison of methods. For example, when using specular reflectance spectra the range of predicted values was much wider, with numbers ranging from .01 to .90 for Gram positive species and from -.44 to –2.10 for Gram negative bacteria. In addition, there were no rejected standards in the ATR model, while the use of specular reflectance spectra resulted in 3 rejected Gram positive standards and one rejected Gram negative standard. In the ATR model the Gram stain property correlated with the spectra at a multiple correlation coefficient of .9502, much higher than the previous .7917 obtained using specular reflectance. Overall, comparing the correlation coefficients, the Gram stain prediction groupings, and the number of rejected standards, the PCR method using ATR spectra has better prediction capabilities than the same method using specular reflectance spectra.

The fact that a numerical value is obtained for Gram stain when employing the methods of PCR may aid in developing a measure of Gram variability. We performed an experiment to investigate this possibility. Incubation studies were done on our eight Gram positive colonies to see if their Gram stain varied after a longer time in the incubator. The experiment previously run was performed on the bacteria after 24 +/- 2 hours of growth in the incubator, and spectra were obtained. After 48 hours of growth, ATR spectra were obtained again and the previous model used to predict Gram stain property. The Gram stain prediction of the bacteria changes in a negative direction with longer growth time. After 72 hours in the incubator, all of the Gram positive species were predicted to be negative with our model. Table 3 shows how the Gram stain prediction changes after each 24-hour growth period for a Gram positive species and a Gram negative species. This is consistent with the fact that the cell wall of Gram positive bacteria deteriorates with time. As the time of incubation increases, the bacteria will stain pink rather than purple, as Gram positive normally stain, showing this Gram variability.

Another experiment performed was expanding the original set of 31 bacteria to 44 species. We added 13 Gram negative bacteria (Gram positive species are harder to obtain and cultivate) to test the effect of a larger, more varied calibration set on Gram stain differentiation. The results show that all of the Gram positive species had positive predicted values, and the Gram negative species had negative predicted values for Gram stain property. Table 4 shows the expanded ATR set. The range of predicted values is slightly tighter in the expanded set, with positive values ranging from .30 to 1.30 and negative values ranging from -.71 to –1.4. There were no rejected standards in the

expanded model, the same as in the original model. The multiple correlation of the Gram stain property with the new set was .9520, slightly higher than the multiple correlation of .9502 with the original set of spectra. Expanding the model to include more standards produced slightly better prediction capabilities for Gram stain using PCR on the ATR spectra, as seen in the correlation coefficients and predicted value groupings.

A last experiment we performed was to investigate the effect of media on the predicting capabilities of our model. All 44 bacteria species were grown on blood agar rather than T-soy for comparison purposes to see if the variability of growth due to the media would change the correlation of Gram stain property. PCR analysis was again used, and two new models were built, one with the averaged spectra of the 31 original bacteria and one with the expanded set of 44 species, all grown on the blood. In the analysis of the model with the original 31 bacteria grown on blood, all the Gram positive colonies had positive predicted values, and all the Gram negative colonies had negative predicted values. In the expanded model of bacteria grown on blood agar one mistake was found: one of the Gram positive species had a negative predicted value. This could be due to slightly altered growth conditions, and it could also be due to the amount of variability with a different media compounded with such a large set. Figure 3 shows the ATR spectra of *Enterococcus faecalis* and *Shigella flexneri*, both on blood agar. The spectra show the amide I and amide II bands (previously bands A and B) merge into one, perhaps because of the high water content of the bacteria on the blood. There are more low frequency absorptions apparent in the region between 1500 cm^{-1} and 1300 cm^{-1} of these spectra than in the ATR spectra of the same colonies on T-soy agar. This may be due to the composition of the blood agar. Blood and T-soy agar are both complex

media by definition, meaning they are made up of a variety of nutrients, most of which are similar in both. Blood is an extra protein-rich component added to this basic nutrient agar, though, which changes the chemical make-up. Therefore we assign the newly apparent lower frequency bands to protein absorptions, probably side-chain vibrations (9).

The extra observable features in the lower frequency of the ATR spectra of bacteria grown on blood are consistent with the PCR analysis, which extracted 10 factors, or principal components, in the models built from the bacteria grown on blood and only 9 factors in the models made from bacteria grown on T-soy agar. In all of the models, made of bacteria grown on T-soy and blood, only 7 factors correlated with Gram stain property. The model of the 31 original colonies grown on blood agar has a high correlation of the 7 factors with Gram stain property, the multiple correlation coefficient being .9595. The spread of values for Gram positive species is tighter than in the original model of bacteria on T-soy agar, with values ranging from .59 to 1.21. The spread of values for Gram negative species is not as tight, though, with values ranging form -.50 to −1.46. The expanded model built from bacteria grown on blood did not have as high a correlation with the 7 factors, the multiple correlation being .8960. The spread of values was also not as tight as the corresponding expanded model of colonies on T-soy agar. The Gram positive species had predicted values ranging from -.07486 to 1.208 (due to the one mistake), and the Gram negative species had predicted values ranging from -.3883 to −1.436. This larger spread could be due to the amount of variability in the set, resulting from the blood agar media compounded with the larger number of standards. The blood models using ATR spectra still show better potential for predicting Gram stain correlation

and for classifying bacteria than the method using specular reflectance spectra. Also, our experiment with blood agar shows that bacteria grown on different media can be used as standards in the PCR analysis and have good differentiation and prediction capabilities.

Our research on ATR microspectroscopy of bacteria shows that the ATR objective does produce a better correlation between spectra and the Gram stain property than the methods developed using specular reflectance spectra or transmittance spectra, and more than one agar can be used as growth media with positive results. Further avenues of study with different media could generate new models to test the differentiation capabilities of PCR, and these models could be useful in predicting Gram stain and classifying bacteria. Also, we have recently obtained more sophisticated PC software that will allow more data. A future study will be to use this software to perhaps combine the T-soy and blood spectra, and it can allow us to rotate factors to try and determine the actual contributions from the many possible bacteria components.

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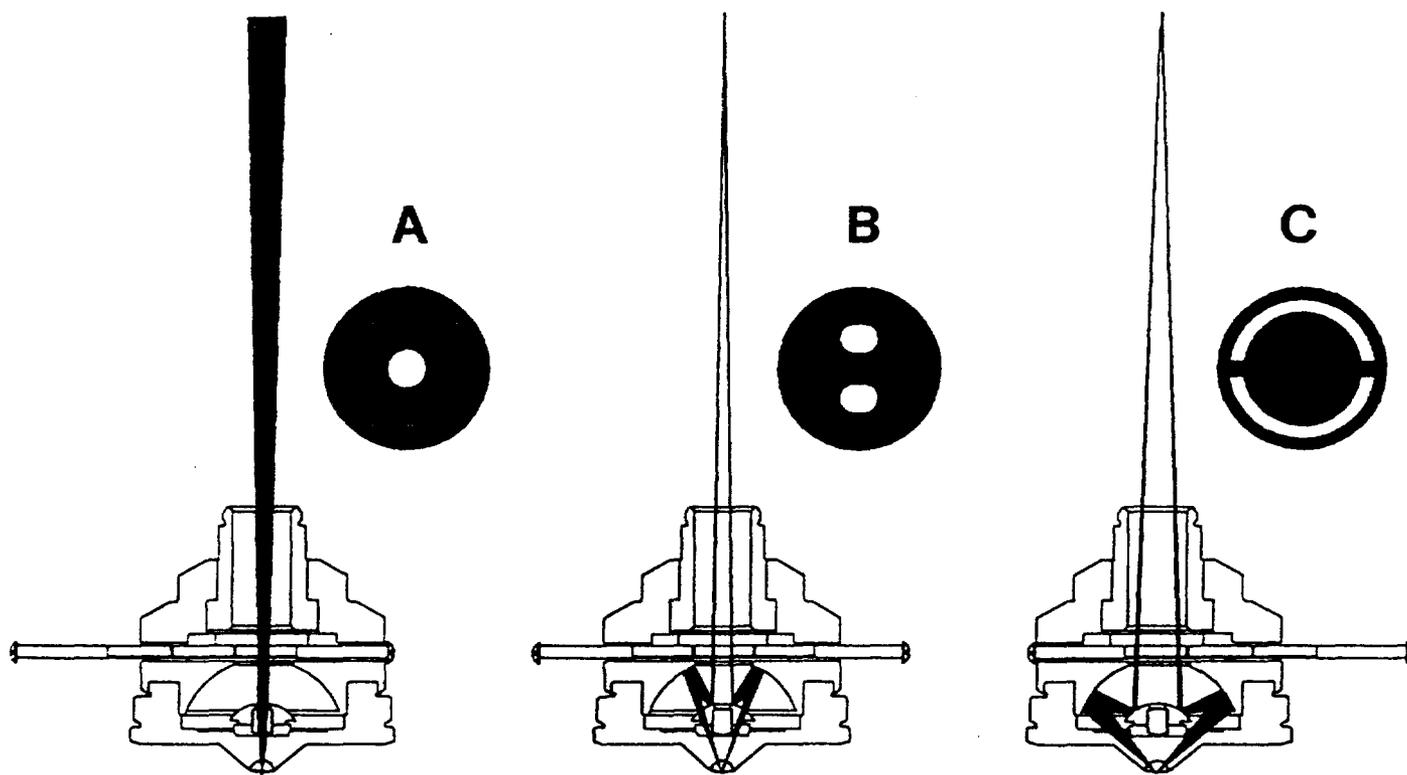
Figure 1 Diagram of the three modes of the ATR objective and light paths through each. The three modes are : Survey mode (A), Contact mode (B), and ATR mode (C).
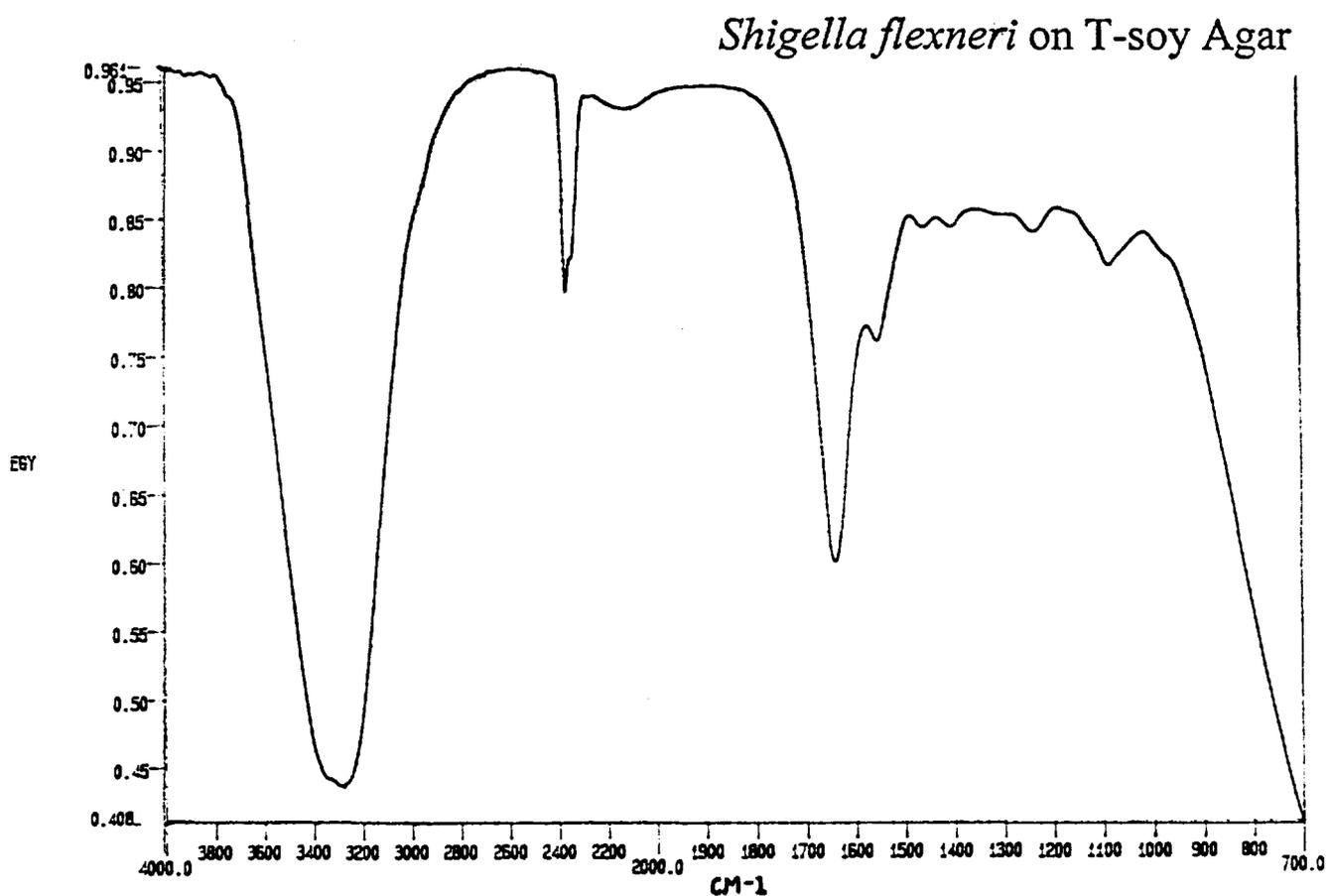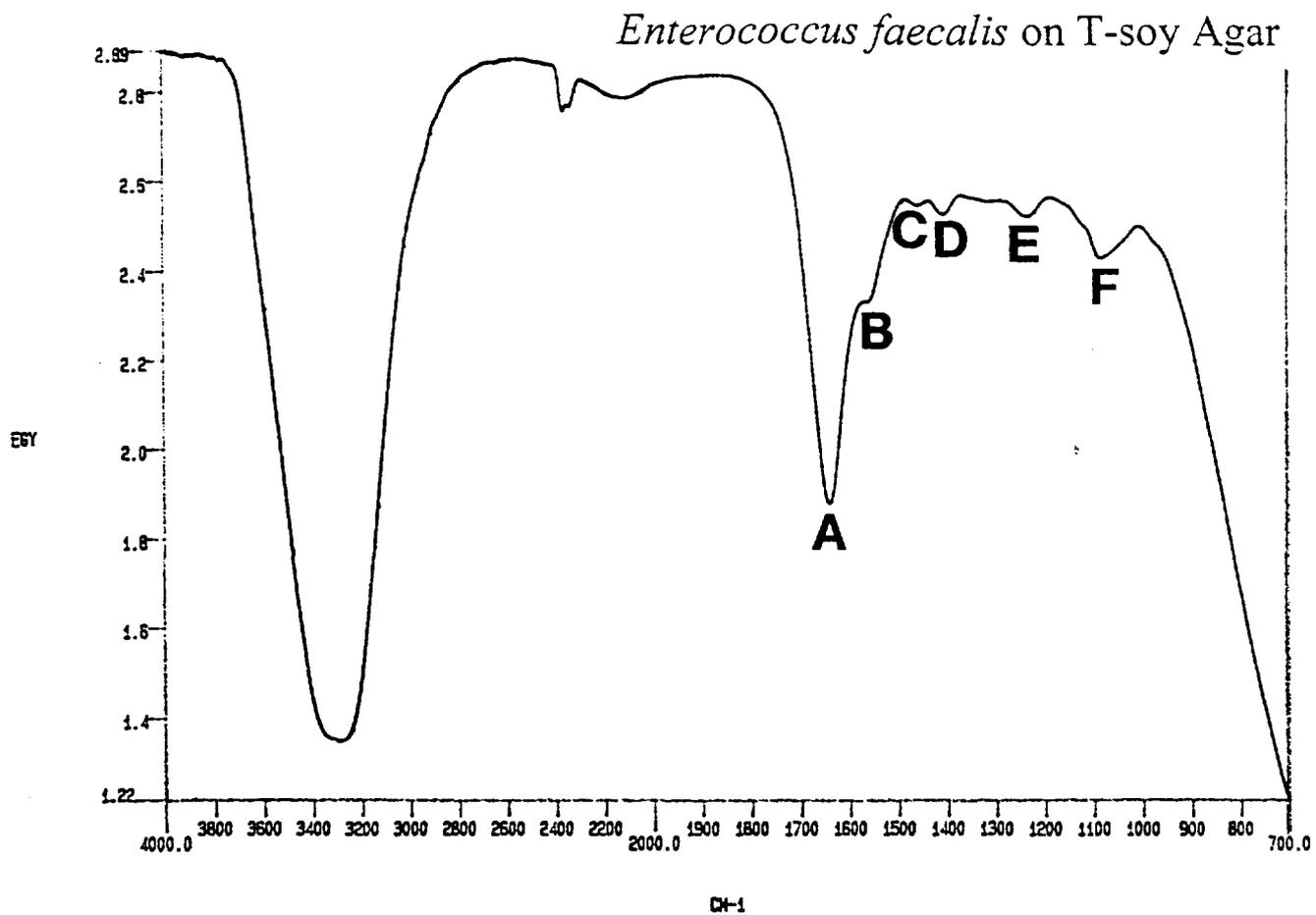
Figure 2 ATR spectra of *Enterococcus faecalis* (top), a Gram positive species, and *Shigella flexneri* (bottom), a Gram negative species, both grown on T-soy agar.

Table 1

Comparison of Band Frequencies

		Band A	Band B	Band C	Band D	Band E	Band F
Enterococcus faecalis	Specular	1639.6	1552.0	1454.9	1402.9	1236.0	1082.8
	ATR	1639.4	1559.9	1458.0	1404.4	1233.2	1080.2
Shigella flexneri	Specular	1634.2	1544.7	1454.3	1400.3	1241.1	1084.5
	ATR	1639.6	1552.7	1459.0	1403.0	1235.6	1083.2

Table 2   *Gram Stain Predictions*

Bacteria Species	Actual Gram Stain	Predicted Gram Stain
Bacillus cereus	1	.77
Bacillus megatherium	1	1.2
Bacillus subtilis	1	.68
Corynebacterium pseudodiptheriticum	1	.21
Enterococcus faecalis	1	.35
Staphylococcus aureus	1	1.1
Staphylococcus epidermidis	1	1.2
Staphylococcus saprophyticus	1	1.3
Citrobacter freundii	-1	-0.89
Enterobacter aerogenes	-1	-1.1
Enterobacter agglomerans	-1	-1.0
Enterobacter cloacae	-1	-0.74
Edwardsiella tarda	-1	-0.84
Escherichia coli	-1	-1.2
Klebsiella pneumoniae	-1	-0.57
Proteus mirabilis	-1	-0.93
Proteus morgani	-1	-0.70
Proteus vulgaris	-1	-1.2
Proteus inconstans	-1	-0.87
Providencia rettgeri	-1	-1.2
Pseudomonas aeruginosa	-1	-1.0
Salmonella arizonae	-1	-0.88
Salmonella indiana	-1	-0.86
Salmonella typhi	-1	-0.81
Salmonella typhimurium	-1	-0.97
Serratia liquifaciens	-1	-1.2
Serratia marcescens	-1	-0.66
Shigella boydii	-1	-0.99
Shigella dysenteriae	-1	-0.85
Shigella flexneri	-1	-1.0
Shigella sonnei	-1	-1.3

Table 3

Gram Variability Studies

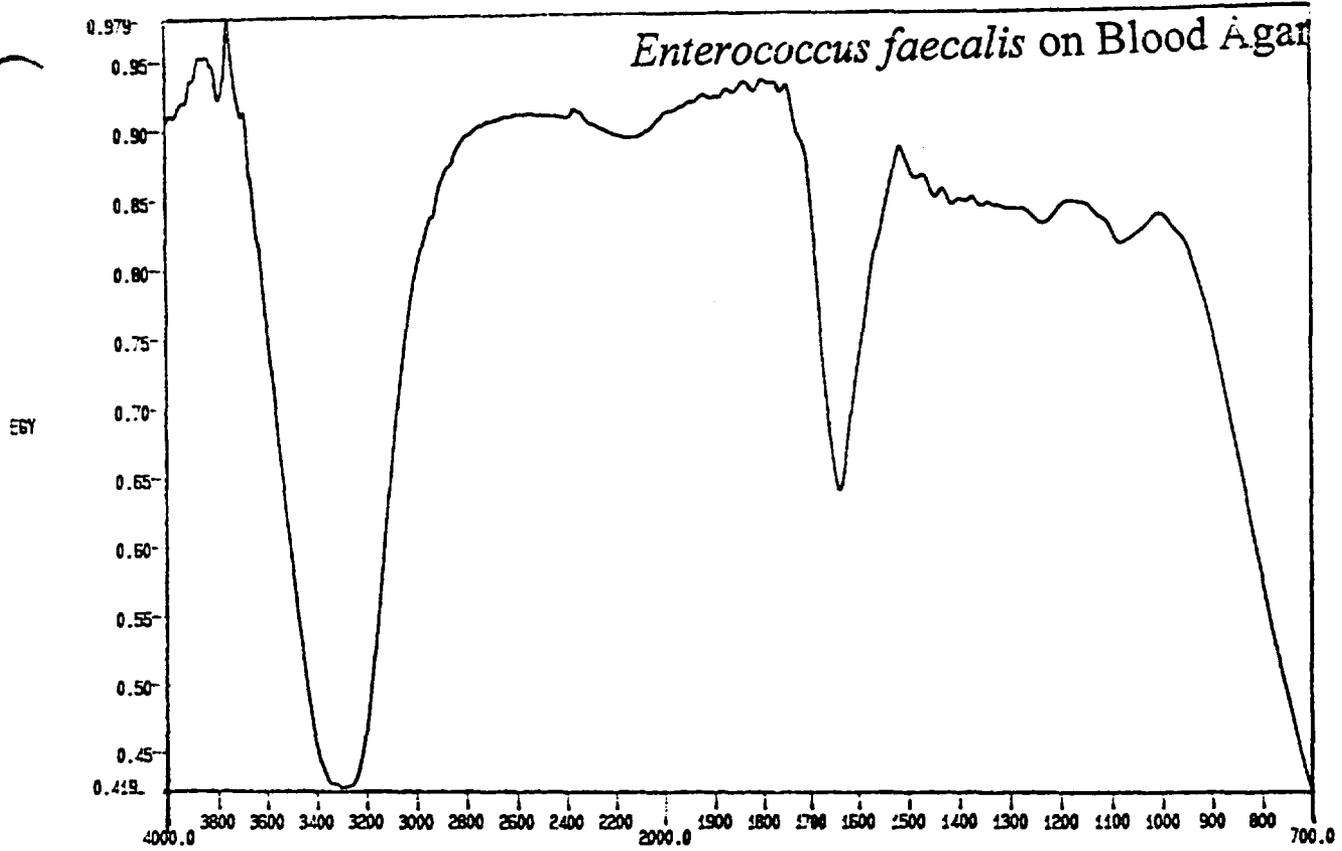|	Day 1	Day 2	Day 3
Bacillus cereus	1.10	.21	-1.30
Staph. epidermidis	1.3	-0.05	-1.60

All gram positive species went from positive values to negative values after three days of incubation.
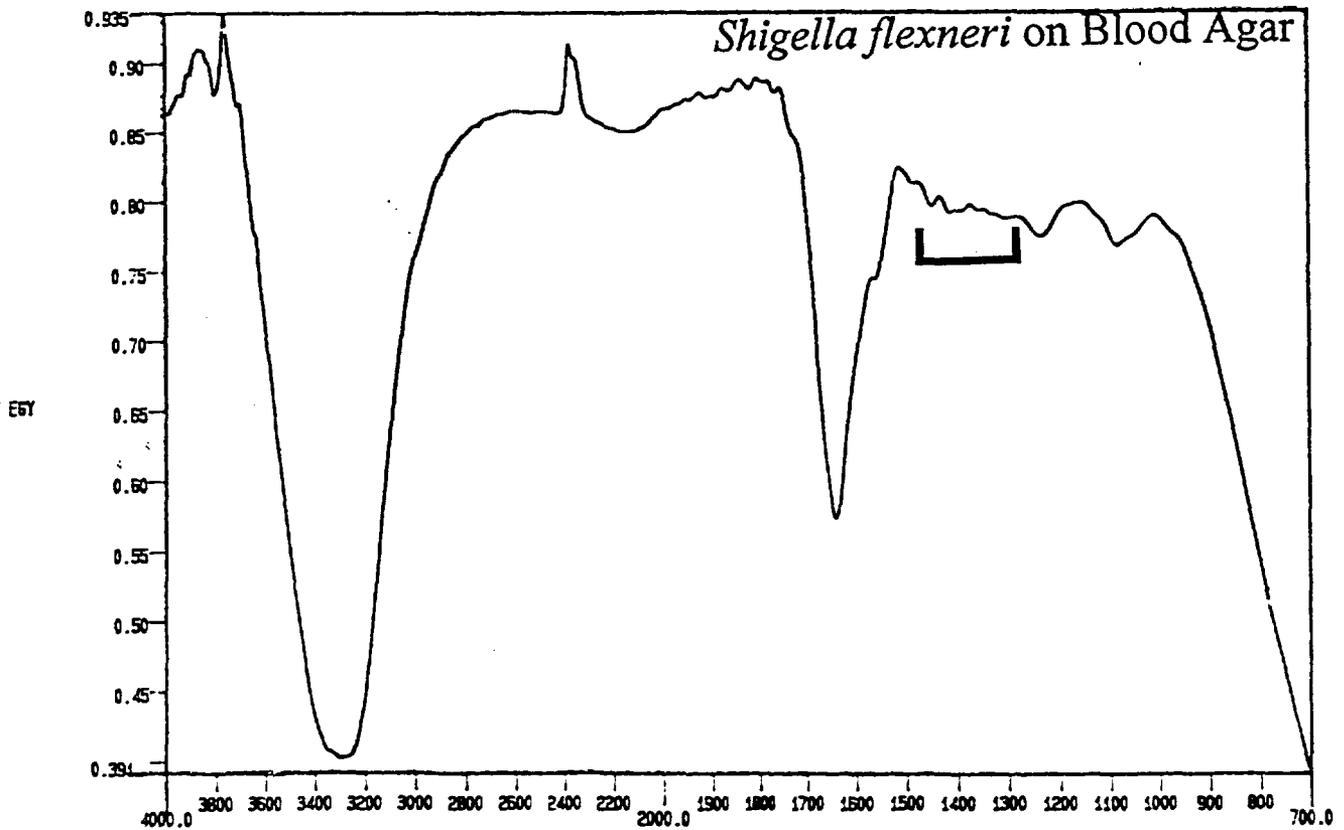
Table 4   *Gram Stain Prediction*

Bacteria Species	Actual Gram Stain	Predicted Gram Stain
Bacillus cereus	1	.69
Bacillus megatherium	1	1.1
Bacillus subtilis	1	.98
Corynebacterium pseudodiptheriticum	1	.31
Enterococcus faecalis	1	.30
Staphylococcus aureus	1	1.3
Staphylococcus epidermidis	1	.75
Staphylococcus saprophyticus	1	1.3
Citrobacter freundii	-1	-1.0
Enterobacter aerogenes	-1	-1.2
Enterobacter agglomerans	-1	-1.1
Enterobacter cloacae	-1	-.83
Edwardsiella tarda	-1	-.89
Escherichia coli	-1	-1.2
Klebsiella pneumoniae	-1	-.78
Proteus mirabilis	-1	-.76
Proteus morgani	-1	-.73
Proteus vulgaris	-1	-1.2
Proteus inconstans	-1	-.75
Providencia rettgeri	-1	-1.2
Pseudomonas aeruginosa	-1	-.86
Salmonella arizonae	-1	-.93
Salmonella indiana	-1	-.98
Salmonella typhi	-1	-.92
Salmonella typhimurium	-1	-1.1
Serratia liquifaciens	-1	-1.2
Serratia marcescens	-1	-.71
Shigella boydii	-1	-.74
Shigella dysenteriae	-1	-.95
Shigella flexneri	-1	-.99
Shigella sonnei	-1	-1.4
Yersinia spp.	-1	-1.1
Salmonella tennessee	-1	-1.2
Salmonella pullorum	-1	-.87
Salmonella paratyphi	-1	-.76
Salmonella gallinarum	-1	-.95
Pseudomonas stutzeri	-1	-.95
Pseudomonas putrefaciens	-1	-1.0
Pseudomonas pseudoalcaligenes	-1	-.76
Pasteurella multocida	-1	-1.3
Moraxella osloeusis	-1	-1.0
Klebsiella spp.	-1	-.75
Enterobacter hafnia	-1	-.98
Citrobacter diversus	-1	-.94

Figure 3 ATR spectra of *Enterococcus faecalis* (top) and *Shigella flexneri* (bottom), both grown on blood agar.